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Structure of cytochrome a_3–Cu_{a_3} couple in cytochrome *c* oxidase as revealed by nitric oxide binding studies

(electron paramagnetic resonance/antiferromagnetic coupling/heme proteins/copper proteins/ferroheme–NO)

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ABSTRACT **The addition of NO to oxidized cytochrome *c* oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) causes the appearance of a high-spin heme electron paramagnetic resonance (EPR) signal due to cytochrome a_3. This suggests that NO coordinates to $Cu_{a_3}^{+2}$ and breaks the antiferromagnetic couple by forming a cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO complex. The intensity of the high-spin cytochrome a_3 signal depends on the method of preparation of the enzyme and maximally accounts for 58% of one heme. The effect of N_3^- on the cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO complex is to reduce cytochrome a_3 to the ferrous state, and this is followed by formation of a new complex that exhibits EPR signals characteristic of a triplet species. On the basis of optical and EPR results, a NO bridge between cytochrome a_3^{+2} and $Cu_{a_3}^{+2}$ is proposed—i.e., cytochrome a_3^{+2}–NO–$Cu_{a_3}^{+2}$. The half-field transition observed at g = 4.34 in the EPR spectrum of this triplet species exhibits resolved copper hyperfine splittings with $|A_\parallel| = 0.020\ cm^{-1}$, indicating that the $Cu_{a_3}^{+2}$ in the cytochrome a_3^{+2}–NO–$Cu_{a_3}^{+2}$ complex is similar to a type 2 copper site.**

Mitochondrial cytochrome *c* oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) catalyzes the four-electron reduction of oxygen to water in the last step of cellular respiration. The functional form of the protein is known (1) to contain two copper and two iron atoms, the latter in the form of heme A. The electron paramagnetic resonance (EPR) spectrum of oxidized cytochrome *c* oxidase (2, 3) exhibits a low-spin Fe(III) signal which has been assigned to cytochrome *a* and an unusual g = 2 signal attributed to the Cu_a center. No evidence has been found to suggest that either of these metal centers binds externally added ligands. The remaining two metal centers, cytochrome a_3 and Cu_{a_3}, appear to be EPR silent in the oxidized protein. It has been shown by magnetic susceptibility measurements (4) that cytochrome a_3 [Fe(III), S = 5/2] and Cu_{a_3} [Cu(II), S = 1/2] are strongly antiferromagnetically coupled to form an S = 2 spin system which does not exhibit an EPR signal with conventional EPR instrumentation.

Cytochrome a_3 has been extensively investigated through ligand binding studies, because this heme center is known to bind CN^- (5), CO (6), S^{-2} (7), and NO (8). The binding of N_3^- (9), F^- (10), and formate (11) to the oxidized protein produces smaller changes in the optical spectrum than are observed for the binding of these ligands to other ferric hemoproteins and these ligands may actually interact with Cu_{a_3} rather than with cytochrome a_3.

This paper is concerned with the interaction of NO with cytochrome *c* oxidase. It has previously been shown that the addition of NO to reduced cytochrome *c* oxidase gives rise to a NO–ferrocytochrome a_3 complex (8, 12) which is EPR visible. This EPR signal exhibits a nine-line superhyperfine pattern that has been shown to arise from two inequivalent nitrogens, the NO nitrogen and an endogenous nitrogen bound axially to cytochrome a_3 (8, 12). The presence of an endogenous axial nitrogen ligand on cytochrome a_3 has led to the suggestion (13) that a histidine imidazole bridges cytochrome a_3 and Cu_{a_3}. It has been proposed that this bridging imidazole facilitates the strong exchange coupling of the two metal centers in oxidized cytochrome *c* oxidase.

NO is also known to bind to certain ferric hemoproteins. The ferricytochrome *c* peroxidase–NO complex has been shown (14) to be EPR silent from 4 to 296 K; the ferricytochrome *c*–NO complex has been shown by magnetic susceptibility measurements to be diamagnetic (15). In addition, NO can react with both oxidized and reduced copper proteins (16, 17). In particular, the reaction of NO with oxidized ceruloplasmin is reversible after a short incubation time (16), suggesting the formation of a reversible copper–NO complex that is apparently EPR silent. However, the binding of NO to oxidized cytochrome *c* oxidase has not been reported. We have found that, when NO is added to oxidized cytochrome *c* oxidase, two distinct complexes are formed depending on whether or not N_3^- is present. These results have allowed us to define better the structure of the cytochrome a_3–Cu_{a_3} couple.

MATERIALS AND METHODS

Beef heart cytochrome *c* oxidase was isolated by the procedures of Hartzell and Beinert (18), Yu *et al.* (19), and Rosén (20). All experiments described were carried out with the enzyme isolated by the procedure of Hartzell and Beinert unless otherwise stated. The purified enzyme was stored at −85°C until used. The preparations contained 9–11 nmol of heme A per mg of protein as measured by the pyridine hemochromagen assay (21). The purified protein was dissolved in 0.5% Tween 20/50 mM Tris·HNO_3, pH 7.4, to a protein concentration of approximately 50 mg/ml with the exception of the Yu *et al.* preparation which was dissolved in 0.5% Na cholate/50 mM phosphate brought to pH 7.4 with HCl. The protein concentrations were then determined by the method of Lowry *et al.* (22).

The protein–NO samples were prepared by the addition of NO (Matheson) to the anaerobic† protein (with or without added ligands) to a pressure of 1 atm (101.3 kilopascals), unless otherwise stated. The samples were made anaerobic by three cycles of evacuation and flushing with argon. These samples were allowed to equilibrate with NO for 10 min at 277 K before being frozen at 77 K. KF, KCN, or NaN_3 was added to the oxidized protein–NO complex from a sidearm on the EPR tube or optical cuvette to yield a final concentration of 100 mM, unless otherwise stated.

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Abbreviation: EPR, electron paramagnetic resonance.

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† Anomalous results are observed if the sample is not completely anaerobic before the addition of NO.

The EPR spectra were recorded on a Varian E-line Century series X band spectrometer equipped with an Air Products Heli-Trans low-temperature system. Optical measurements were carried out at room temperature on a Beckman Acta CIII spectrometer.

The intensity of the high-spin cytochrome a_3 EPR signals was determined relative to an external myoglobin standard and also relative to the low-spin cytochrome *a* EPR signal. The high-spin EPR signals were integrated by the method of Aasa *et al.* (3). The low-spin cytochrome *a* EPR signal was integrated by the method of Aasa and Vänngård (23) using the g = 3.0 component to determine the total area. The low-spin cytochrome *a* EPR signal has been shown to correspond to 100% of one heme (3), and on this basis the low-spin heme EPR signal was used as an internal standard. The high-spin cytochrome a_3 EPR signal intensities determined by using a myoglobin standard were found to be independent of temperature, thus indicating that the zero-field splitting parameters (*D*) are nearly equal for the two high-spin ferric hemes. After correction for the distribution of population among the spin sublevels of the high-spin ferric heme, the cytochrome a_3 EPR intensities determined by using the internal cytochrome *a* standard were found to agree with those determined by using the myoglobin standard to within 10%.

RESULTS

Oxidized Cytochrome *c* Oxidase + NO. The EPR spectrum of the oxidized cytochrome *c* oxidase–NO complex is shown in Fig. 1. There were no changes in the intensity or position of the low-spin cytochrome *a* or the Cu_a center EPR signals upon the addition of NO. However, a new rhombic high-spin heme EPR signal, which corresponded to as much as 58% of one heme, was observed although the intensity of this high-spin heme EPR signal did depend on the method of preparation of the enzyme (Table 1). Because the cytochrome *a* EPR signal remained unchanged, the new high-spin heme EPR signal can be attributable only to cytochrome a_3. This rhombic high-spin heme signal has $g_x = 6.16$ and $g_y = 5.82$. The position of g_z is obscured by the Cu_a signal. The temperature dependence of the high-spin cytochrome a_3 EPR signal indicates that the zero-field splitting parameter (*D*) is about 6 cm^{-1}. The broad signal centered at about g = 1.97 in the oxidized cytochrome *c* oxidase–NO EPR spectrum was also seen in the spectrum of the buffer plus NO sample (Fig. 1) and is presumed to be matrix-bound paramagnetic NO.

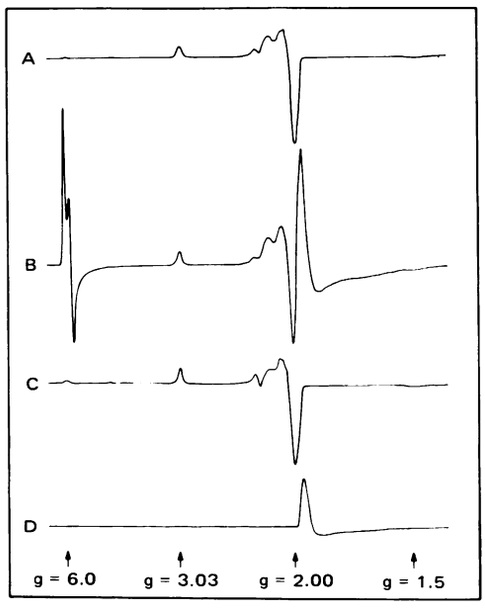

FIG. 1. The EPR spectra of native oxidized cytochrome *c* oxidase (A), NO added to A to a pressure of 723 mm Hg (B), NO removed from B (C); and NO added to the buffer only (D). The signals at g = 3.03, 2.21, and 1.5 are due to cytochrome *a* and the signals at g = 2.18, 2.03, and 1.99 are due to Cu_a. Conditions: temperature, 7 K; microwave power, 0.2 mW; modulation amplitude, 10 G; microwave frequency, 9.16 GHz.

Table 1. Fraction of high-spin cytochrome a_3 EPR signal observed for various preparations of oxidized cytochrome *c* oxidase in presence of NO at 1 atm

Preparation	Fraction observed
Hartzell and Beinert no. 1	0.58
Hartzell and Beinert no. 1 + 100 mM F^-	0.61
Hartzell and Beinert no. 1, "oxygenated"	<0.05
Hartzell and Beinert no. 2	0.26
Yu *et al.* no. 1	<0.05
Yu *et al.* no. 1 + 10 mM F^-	0.26
Rosén no. 1	<0.05

The binding curve of NO to oxidized cytochrome *c* oxidase (Fig. 2) demonstrates that the intensity of the high-spin cytochrome a_3 signal is dependent on the NO pressure. The pressure of NO corresponding to the appearance of 50% of the observed high-spin cytochrome a_3 EPR signal was about 65 mm Hg. This process of NO binding to oxidized cytochrome *c* oxidase is reversible, because the high-spin cytochrome a_3 EPR signal disappeared with removal of NO from the sample (Fig. 1).

We found that the optical spectrum of oxidized cytochrome *c* oxidase remained unchanged upon the addition of NO (Fig. 3). The lack of any effect by NO on the optical spectrum of oxidized cytochrome *c* oxidase indicates that no NO–heme interaction occurs. These optical results in conjunction with the EPR data suggest the formation of a cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO complex.

The addition of NO to oxidized cytochrome *c* oxidase prepared by the method of Yu *et al.* or the method of Rosén did not produce a large high-spin cytochrome a_3 EPR signal. In this regard, we found that the Hartzell and Beinert preparation of the enzyme, which exhibited a large high-spin heme EPR signal upon the addition of NO to the resting oxidized protein, *did not* exhibit a high-spin heme EPR signal when NO was added to

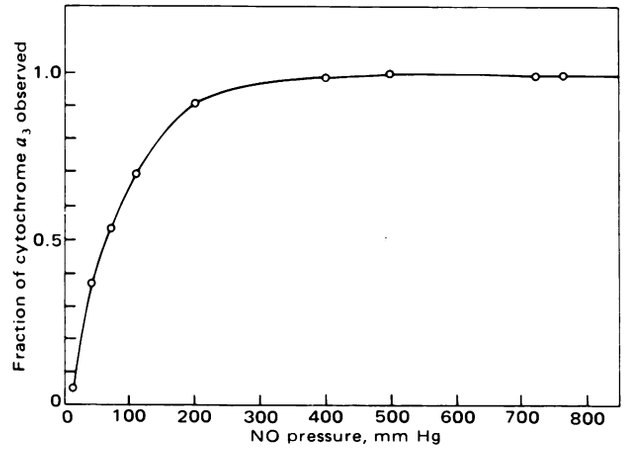

FIG. 2. Binding curve of NO to oxidized cytochrome *c* oxidase. The fraction of cytochrome a_3 observed is scaled such that 100% refers to the maximal high-spin heme EPR signal intensity observed. The maximal signal intensity was found to depend on the method of preparation of the enzyme (Table 1). The pressure of NO represents the total pressure above the sample.

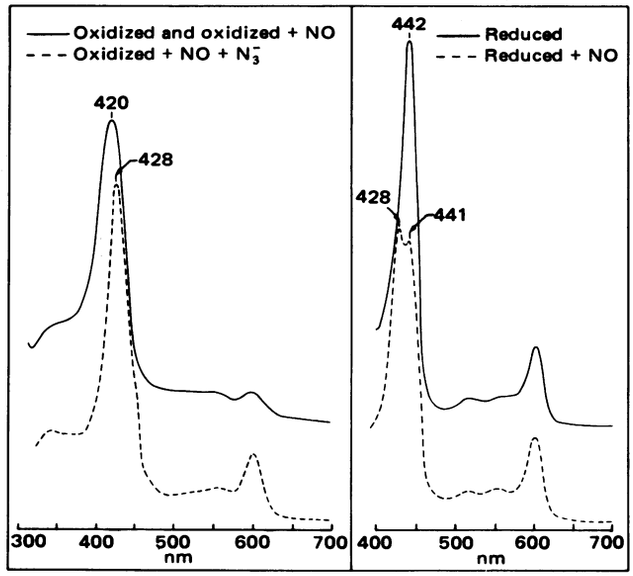

FIG. 3. The optical spectra of oxidized (*Left*) and reduced (*Right*) cytochrome *c* oxidase in the presence and absence of NO and N_3^-.

this enzyme in the "oxygenated" state (24). Inasmuch as it is believed that "oxygenated" cytochrome *c* oxidase exists in a conformation different from that of the oxidized resting enzyme (24), these results suggest that the nature of the interaction of NO with oxidized cytochrome *c* oxidase may depend on the conformational state of the enzyme.

Oxidized Cytochrome *c* Oxidase–NO + KF. In order to determine whether other exogenous ligands compete with NO for the same binding site(s) in the protein, we examined the addition of F^- to the oxidized cytochrome *c* oxidase–NO complex. The addition of 100 mM KF to the oxidized cytochrome *c* oxidase-NO complex produced a change in the shape of the high-spin cytochrome a_3 EPR signal (Fig. 4) with no change in the total intensity. The signal observed in the presence of F^- appears to be due to a superposition of a nearly axial high-spin heme EPR signal and the more rhombic high-spin heme EPR signal observed in the cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO complex. Therefore, it appears that F^- and NO can bind simultaneously to the oxidized protein, and the resulting complex exhibits a nearly axial high-spin heme EPR signal.

Because the fluoride-bound Hartzell and Beinert preparation of the enzyme exhibited a new high-spin heme EPR signal upon the addition of NO, we also examined the addition of NO to the fluoride-bound Yu *et al.* preparation of the protein. In the presence of 10 mM F^- and NO, the protein isolated by the method of Yu *et al.* exhibited a nearly axial high-spin heme EPR signal that accounted for 26% of one heme (Fig. 4). As observed for the Hartzell and Beinert preparation of the protein, the NO complex in the presence of F^- was reversible upon removal of NO from the sample. These observations further suggest that the interaction of NO with oxidized cytochrome *c* oxidase depends on the conformation of the protein and that F^- is capable of inducing a conformation in the Yu *et al.* preparation of the enzyme that yields a high-spin cytochrome a_3 EPR signal upon the addition of NO.

Oxidized Cytochrome *c* Oxidase–NO + KCN. Upon addition of 5 mM CN^- to the oxidized protein–NO complex, the high-spin heme EPR signal observed in the cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO complex disappeared. The oxidized cytochrome *c* oxidase–NO+KCN EPR spectrum exhibited normal cytochrome *a* and Cu_a EPR signals. In addition, a signal was observed at g = 3.5 which is characteristic of a cytochrome

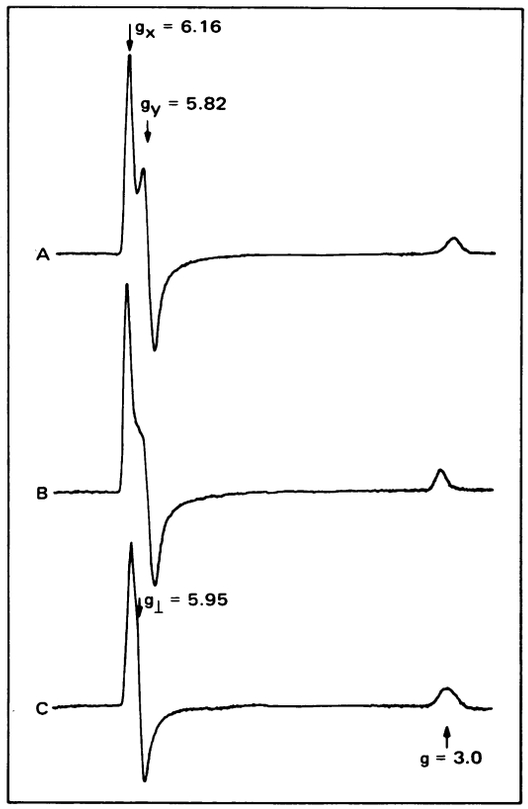

FIG. 4. The EPR spectra of oxidized cytochrome *c* oxidase prepared by the method of Hartzell and Beinert with NO added (A); KF added to A to give a concentration of 100 mM (B); and oxidized cytochrome *c* oxidase prepared by the method of Yu *et al.* with 10 mM KF and NO (C). Conditions: temperature, 9 K; microwave power, 0.05 mW; modulation amplitude, 12.5 G; microwave frequency, 9.24 GHz.

a_3^{+3}–CN^- species and which accounted for approximately 10% of one heme. Furthermore, the order of addition of CN^- and NO to the oxidized enzyme did not affect these results. Because a large fraction of the cytochrome a_3 EPR signal intensity disappeared upon the addition of CN^- to the oxidized protein–NO complex, we conclude that CN^- displaces a large fraction of the NO molecules from oxidized cytochrome *c* oxidase. However, we found that the g = 3.5 signal did not disappear upon removal of NO from the sample. Therefore, at this point we cannot distinguish between the possibility that CN^- and NO are simultaneously binding to cytochrome a_3^{+3} and $Cu_{a_3}^{+2}$, respectively, or that CN^- facilitates the reduction of $Cu_{a_3}^{+2}$ in approximately 10% of the enzyme molecules in the presence of NO.

Oxidized Cytochrome *c* Oxidase–NO + N_3^-. The addition of N_3^- to the oxidized protein–NO complex resulted in a dramatic change in the optical spectrum (Fig. 3). The Soret band shifted 8 nm to lower energy and narrowed substantially; the α-band increased 2-fold in intensity. The positions of the Soret bands for various cytochrome *c* oxidase species are compared in Table 2. It is clear from this spectral comparison that the effect of N_3^- together with NO is to reduce cytochrome a_3^{+3}, followed by the binding of NO to cytochrome a_3^{+2}. We have obtained no evidence that this process affects cytochrome a^{+3}.

A possible scheme for the reduction of cytochrome a_3 is

$$\text{Cytochrome } a_3^{+3} + N_3^- + NO \rightarrow \text{cytochrome } a_3^{+2} + N_2O + N_2. \quad [1]$$

Table 2. Positions of Soret bands for various cytochrome *c* oxidase species

Species	Probable state	Soret position, nm: Cytochrome *a*	Soret position, nm: Cytochrome a_3	Soret observed, nm
Oxidized cytochrome *c* oxidase	a^{+3}, a_3^{+3}	426*	414*	420
Reduced cytochrome *c* oxidase	a^{+2}, a_3^{+2}	444*	443*	442
Oxidized cytochrome *c* oxidase + NO	a^{+3}, a_3^{+3}	426†	414‡	420
Reduced cytochrome *c* oxidase + NO	a^{+2}, a_3^{+2}–NO	444†	428	428; 441
Oxidized cytochrome *c* oxidase + N_3^- + NO	a^{+3}, a_3^{+2}–NO	426†	428	428

* See ref. 25.

† It is assumed that the cytochrome *a* Soret band is not dependent on the oxidation or ligation state of cytochrome a_3.

‡ Cytochrome a_3^{+3} is assumed to be unperturbed by NO because the optical spectrum is virtually unchanged upon the addition of NO to oxidized cytochrome *c* oxidase.

This scheme predicts the production of N_2O, which we have detected by mass spectroscopy. The substitution of ^{15}NO for ^{14}NO resulted in a N_2O parent peak located at $M/e = 45$, indicating that the nitrogen from NO appears in the N_2O molecule after the reaction. No N_2O was detected in the absence of cytochrome *c* oxidase, indicating that reaction 1 probably occurs at the ligand binding site(s).

The formation of the above cytochrome a_3^{+2}–NO complex did not alter the low-spin cytochrome a^{+3} or the Cu_a center EPR signals (Fig. 5). As expected, the high-spin heme EPR signal observed for the cytochrome a_3^{+3}–Cu_{a3}^{+2}–NO complex disappeared upon the addition of N_3^-. However, no EPR signals typical of NO–ferrohemoproteins (8, 12, 14) were observed for our cytochrome a_3^{+2}–NO complex. Instead, *new* EPR signals appeared near g = 2 and at g = 4.34. These results were observed for cytochrome *c* oxidase prepared by the method of Hartzell and Beinert as well as for that prepared by the method of Yu *et al.*

EPR transitions near g = 2 ($\Delta M_s = 1$) and g = 4 ($\Delta M_s = 2$) are characteristic of a triplet species with a small zero-field splitting. Accordingly, we have attributed the new EPR signals that we observed near g = 2 and at g = 4.34 to a triplet species. The $\Delta M_s = 2$ transition that we observed exhibits a four-line hyperfine pattern with a splitting of 97 G due to a copper nucleus ($|A_\parallel| = 0.020\ cm^{-1}$). This value of $|A_\parallel|$ is indicative of a type 2 copper ion (26). We propose that the triplet signals originate from magnetic coupling of the unpaired electron on the cytochrome a_3^{+2}–NO site with that of the Cu_{a3}^{+2}. In this regard, the temperature dependence of the triplet EPR signal indicates that the two $S = 1/2$ sites are *anti*ferromagnetically coupled with an exchange interaction of about 5 cm^{-1}. The $\Delta M_s \neq 1$ transition near g = 2 indicates that $|D| \approx |3E| \approx 0.07$ cm^{-1}, in which D and E are the axial and rhombic zero-field splitting parameters, respectively (27).

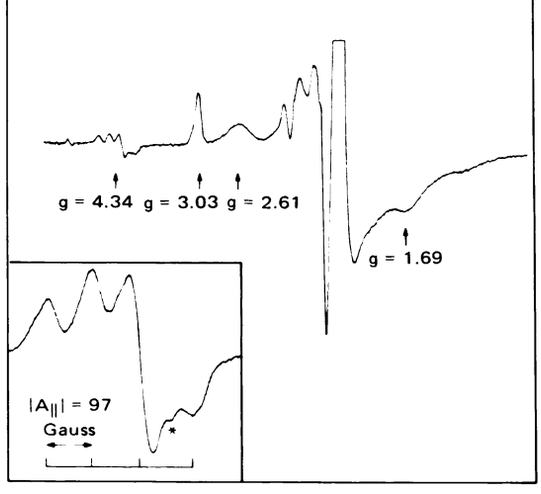

FIG. 5. EPR spectrum of oxidized cytochrome *c* oxidase in the presence of N_3^- and NO. (*Inset*) Magnified view of the half-field transition region. The peak labeled (*) is not part of the triplet signal and is probably due to extraneous ferric iron. Conditions: temperature, 7 K; microwave power, 2 mW (200 mW for the spectrum shown in the *Inset*); modulation amplitude, 10 G; microwave frequency, 9.16 GHz.

We found that the process of NO binding in the presence of N_3^- cannot be reversed, in contrast to that observed in the absence of N_3^-. Furthermore, no changes were observed in the EPR or optical spectra upon the addition of O_2 to the sample. This result indicates that O_2 does not displace NO from this complex.

DISCUSSION

In this work we have studied the interaction of NO with oxidized cytochrome *c* oxidase. Different results have been obtained depending on whether NO interacts with the protein in the absence or presence of N_3^-. In the absence of N_3^-, we obtained evidence for a cytochrome a_3^{+3}–Cu_{a3}^{+2}–NO complex. In the presence of N_3^-, the observations suggest that a bridged cytochrome a_3^{+2}–NO–Cu_{a3}^{+2} complex is formed. In both complexes, the antiferromagnetic coupling between cytochrome a_3^{+3} and Cu_{a3}^{+2} observed in the native oxidized protein is broken.

Cytochrome a_3^{+3}–Cu_{a3}^{+2}–NO Complex. We have shown that NO interacts with oxidized cytochrome *c* oxidase and breaks the antiferromagnetic couple between iron and copper. The resulting complex exhibits a new high-spin heme EPR signal which corresponds to as much as 58% of cytochrome a_3. The optical spectrum of the oxidized protein remains unchanged upon the addition of NO. Taken together, these observations indicate that there is no NO–heme interaction. Moreover, no EPR signals are observed for Cu_{a3}^{+2} in the presence of NO. Therefore, it appears that NO interacts with Cu_{a3}^{+2} to form a cytochrome a_3^{+3}–Cu_{a3}^{+2}–NO complex.

There are two ways in which NO might interact with Cu^{+2} to produce an EPR silent species: (*i*) NO reduces the copper and (*ii*) NO coordinates to the copper to form an exchange coupled complex. We think that situation *ii* is more likely, because the formation of our cytochrome a_3^{+3}–Cu_{a3}^{+2}–NO complex is easily reversible upon the removal of NO. We have observed no signal that can be assigned to a Cu_{a3}^{+2}–NO triplet. Inasmuch as we do observe a high-spin cytochrome a_3 EPR signal which accounts for as much as 58% of one heme, we believe that the Cu_{a3}^{+2} and NO spins are antiferromagnetically coupled with a large exchange interaction relative to kT.

When NO is added to oxidized cytochrome *c* oxidase, we do not observe a signal from 100% of cytochrome a_3. This could

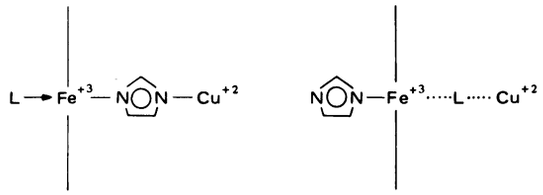

FIG. 6. Two proposed structures for the cytochrome a_3–Cu_{a_3} couple in oxidized cytochrome *c* oxidase. L stands for a possible ligand.

arise because of a conformational heterogeneity of the enzyme molecules which renders only a subpopulation of the enzyme capable of binding NO. However, it is also possible that NO does bind to all of the cytochrome *c* oxidase molecules but that a conformational heterogeneity of the protein allows only a fraction to be observed by EPR spectroscopy. In order to observe a high-spin cytochrome a_3 EPR signal, the interaction of NO with $Cu_{a_3}^{+2}$ must dominate the exchange interaction between the $Cu_{a_3}^{+2}$ and cytochrome a_3^{+3}. It is probable that a change in conformation of the cytochrome a_3–Cu_{a_3} site will alter the magnitude of the exchange interaction between these two metal centers. Thus, it is possible that more than one conformation of NO-bound oxidized cytochrome *c* oxidase exits: one in which the cytochrome a_3–Cu_{a_3} interaction is large with respect to the Cu_{a_3}–NO interaction, resulting in an EPR-silent cytochrome a_3, and another in which the Cu_{a_3}–NO interaction dominates, resulting in an EPR-visible cytochrome a_3. Finally, there exists the possibility that the activity of NO in solution is too low to give complete binding of NO to the enzyme.

Cytochrome a_3^{+2}–NO–$Cu_{a_3}^{+2}$ Complex. We have shown that addition of N_3^- to the cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO complex results in reduction of cytochrome a_3 to the ferrous state, followed by the binding of NO to the cytochrome a_3 moiety. Furthermore, EPR signals typical of a triplet species appear, originating from the thermally accessible $S = 1$ excited state of the antiferromagnetically coupled spins of the NO-bound site and $Cu_{a_3}^{+2}$. Magnetic coupling between the cytochrome a_3^{+2}–NO complex and $Cu_{a_3}^{+2}$ could arise if NO bridges the two metal sites.

Our proposal for the NO bridge is consistent with the known affinity of NO for ferrous cytochrome a_3 (8, 12) as well as the affinity for cupric Cu_{a_3} established here. The evidence (EPR and optical) that neither the removal of NO from the sample nor the subsequent addition of O_2 results in the displacement of NO in the cytochrome a_3^{+2}–NO–$Cu_{a_3}^{+2}$ complex indicates that this bridge is a stable one. In contrast, the binding of NO in the case of the fully reduced cytochrome *c* oxidase–NO complex ($Fe_{a_3}^{+2}$–NO) and in the case of cytochrome a_3^{+3}–$Cu_{a_3}^{+2}$–NO ($Cu_{a_3}^{+2}$–NO) is reversible.

The value of $|A_\parallel|$, 0.020 cm^{-1}, obtained from the hyperfine splittings observed for the $\Delta M_s = 2$ transition of the cytochrome a_3^{+2}–NO–$Cu_{a_3}^{+2}$ triplet species is indicative of a type 2 copper site. In this regard, recent resonance Raman studies (28, 29) have demonstrated that neither copper center in native oxidized cytochrome *c* oxidase is a type 1 copper site. Taken together, these results suggest that the structure of Cu_{a_3} in the native oxidized protein may be similar to a type 2 copper center.

Structure of the Cytochrome a_3–Cu_{a_3} Site in Cytochrome *c* Oxidase. Two models that have been proposed for the cytochrome a_3–Cu_{a_3} site are shown in Fig. 6. In model A, a strongly bound imidazole bridges the iron and the copper sites (13), with the ligand-binding site being the free axial position of the heme iron. In model B, the ligand binding site is between the two metal centers (30). Our evidence that NO bridges the two metal centers in the cytochrome a_3^{+2}–NO–$Cu_{a_3}^{+2}$ complex argues strongly in favor of model B. Model B would also allow formation of a bridged peroxy species as an intermediate in the reduction of molecular oxygen to water by cytochrome *c* oxidase.

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